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Antimicrobial activity screening of endophytic fungi extracts isolated from brown algae *Padina* sp.

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ABSTRACT

This study aims to evaluate the antimicrobial activities of endophytic fungi from marine brown algae *Padina* sp. collected from Nirwana Beach, Padang, West Sumatera, Indonesia. The isolation of endophytic fungi was conducted using dilution method with *Sabouraud Dextrose Agar* (SDA) + *Chloramphenicol* as a growth medium. Nine fungi strains have been isolated from this alga. Purely isolated fungi were cultivated using rice as a medium at room temperature for 3–4 weeks. The secondary metabolite produced by fungi was extracted using ethyl acetate (EtOAc) as a solvent. The antimicrobial activity of EtOAc extracts was tested against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* by using the agar diffusion method. In this research, nine endophytic fungi were isolated from the brown marine algae *Padina* sp. The results of antimicrobial activity screening showed that one fungal isolate ($Nita_3$) was selected as the most active against *S. aureus*, *E. coli*, and *C. albicans* with a diameter of inhibition zone of 20.98 ± 1.56 mm, 17.98 ± 6.58 mm, and 13.60 ± 0 mm, respectively. This selected fungus was identified molecularly as *Trichoderma harzianum*. We conclude that *T. harzianum* can be a source of antimicrobial compounds. However, continuous research is needed to prove its bioactive action.

INTRODUCTION

Secondary metabolite compounds, which are produced by marine-derived fungi, are potential sources of novel antibiotic lead and bioactive compounds. As the success in finding the antibiotic penicillin as a chemical compound produced by the fungus *Penicillium notatum*, various types of fungi are currently seriously studied in the discovery of bioactive compounds (Hawksworth, 1991). Marine-derived endophytic fungi living in the internal tissue without causing a negative effect to their host have proven to be prolific sources of bioactive natural products with unique structures and potent pharmaceutical activities (Bacon and White, 2000; Bugni and Ireland, 2004; Strobel and Daisy, 2003). Fungi have been obtained from virtually every possible marine habitat, including marine plants (algae, driftwood, and mangrove plants), marine invertebrates (sponges, corals, ascidians, and holothurians), and vertebrates (mainly fish). Among them, algae are one of the most prevalent sources of marine-derived fungi for chemical studies. A number of previous studies reported that the diversity of fungi is influenced by the nutrients availability and other physicochemical conditions of the coastal ecosystem (Ashok *et al*., 2015).

Natural products of algal-derived endophytic fungal have been the subject of many chemical reports, especially in the past 10 years. Several new compounds have been isolated and identified with a wide range of biological properties, including anticancer, antibiotic, antiviral, antioxidative, and kinase inhibitory or activated activities (Flewelling *et al*., 2015; Rateb and Ebel, 2011, Singh *et al*., 2014). Varioxepine A, a new alkaloid contained 3H-oxapine isolated from the marine alga-derived fungus *Paecilomyces variotti*, for example, has potent antimicrobial activity against several human pathogenic bacteria with the minimal inhibitory concentration (MIC) values ranging

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from 16 to 46 µg/ml (Zhang *et al*., 2014). In addition, the study of Wong *et al*. (2015) revealed that endophytic fungi from marine algae Padina sp. have potent antimicrobial activity against some pathogenic microbes.

West Sumatera, Indonesia, has a high species diversity of marine algae but there are only a few reports on the screening of West Sumatran marine algae-derived fungi for antibacterial and antifungal activities. The present research was made to study the endophytic fungi isolated from marine algae, *Padina* sp. and its antimicrobial activity against *S. aureus*, *E. coli,* and *C. albicans* by using the agar diffusion method.

MATERIALS AND METHODS

Sample preparation

Padina sp. was collected from Nirwana Beach, Padang, West Sumatera, Indonesia. This marine brown alga was rinsed using sterile sterile sea water and immersed in 70% ethanol for 60–120 seconds and then put in the plastic containing sterile sea water. It was then stored in the icebox and transported to the laboratory for further investigation (Kjer *et al*., 2010).

Isolation of endophytic fungi from marine brown algae Padina sp.

Padina sp. was cut into small pieces. Approximately 10 g of this sample was dispersed in sterile seawater until the volume on Erlenmeyer achieved 100 ml. The mixture was then diluted until 10^{-6} ml. One milliliter of the sample was poured aseptically onto SDA (SDA + *Chloramphenicol*) medium in the Petri dish. The plate was incubated for 5–7 days at temperature 25°C–27°C. Colonies which showed different shapes to others were regarded as different isolates and then purified by the scratch method to obtain pure single isolate (Kjer *et al*., 2010).

Cultivation of pure fungi isolate in the medium of rice

Fungi isolate in the Petri dish was cut 1 × 1 cm, and then cultured in rich medium and incubated at room temperature for 4–6 weeks. The fungi grow maximally when all part of rice was covered by the fungi isolate (Kjer *et al*., 2010).

Extraction of a secondary metabolite of fungi isolate

Pure isolate, which had been grown for 4–6 weeks, was extracted by maceration with ethyl acetate (EtOAc) with the ratio 1:1 for 24 hours and then filtered. The macerate of EtOAc was evaporated with a rotary evaporator to produce the extract of fungi. Then, the extract was ready to be screened for antimicrobial activity (Kjer *et al*., 2010).

Screening for antimicrobial activity

Screening for antimicrobial activity was tested against *S. aureus*, *E. Coli*, and *C. albicans* using the agar diffusion method. The extract was diluted with dimethyl sulfoxide (DMSO) to reach the concentration of 5%, 3%, and 1%. One piece of sterile disk paper (6 mm) was soaked in each of EtOAc extract concentration. DMSO was used for negative control and gentamicin disk as a positive control for bacteria and nystatin disk as a positive control for fungi. Zone of inhibition (mm) was measured after incubation at a temperature of 37°C for 18–24 hours (Bauer *et al*., 1959).

Phytochemical screening of endophytic fungi extract

Phytochemical screening was done for all EtOAc endophytic fungi extracts by using the standard method of Tiwari *et al*. (2011). This method aims to know the alkaloid, phenolic, terpenoid, and steroid compound from each endophytic fungus extract.

Alkaloid detection

0.5 ml diluted hydrochloric acid (10%) was used to dissolve 1 ml extract then filtered. Furthermore, the 1 ml filtrate was added with three to four drops of Mayer reagent. Positive alkaloid will be shown by the yellow color of the filtrate.

Phenolic detection

The extract (1 ml) was dissolved with 0.5 ml diluted hydrochloric acid (10%) then filtered. The filtrate was then added with —three to four drops of ferric chloride solution. Positive phenolic will be shown by the blue color of the filtrate.

Steroid and terpenoid detection

The extract (1 ml) was dissolved with chloroform then filtered. The filtrate was then added with one to two drops of acetic anhydride (99%) and concentrated H_2SO_4. The positive steroid will be shown by the blue or purple color of the filtrate, while terpenoid will be indicated by the red color of the filtrate.

Molecular identification

DNA extraction

DNA extraction was conducted by the following modification method of Atashpaz *et al*. (2010). Once the fungus was grown in the SDA media for 24 hours, its cell biomass was then transferred to the microtube containing 500 µl of lysis buffer. The mixture was centrifuged at 18,000 rpm for 5 minutes at 4°C. The filtrate was removed, and the pellet-contained DNA was dissolved with 50 µl of TE buffer pH 8.0.

Polymerase chain reactions (PCR) amplification and sequence of ITS-encoding gene

PCR thermal cycler (KOD FX Neo, Toyobo, Japan) machine was used for PCR amplification. This PCR following PCR master mix protocol, *KAPA2G Fast Ready mix PCR kit,* with primer internal transcribed spacer (ITS)1 (F 5'- TCC GTA GGT GAA CCT GCG G-3') and primer ITS4 (R 5'- TCC TCC GCT TAT TGA TAT GC-3'). The PCR amplification reaction is as follows: The reaction involved one cycle at 95°C for 5 minutes, followed by 35 cycles with a step of denaturation at 95°C for 30 seconds, step annealing at 55°C for 1 minute, and step extension at 72°C for 1 minute, followed by one cycle at 72°C for 6 minutes (Ferrer *et al*., 2001).

The PCR products were sent to first Base Malaysia for sequencing. The sequences were trimmed and assembled by using the BioEdit V.7.0.5 program. Furthermore, the sequences were subjected to BLAST on NCBI and species identification using the BOLD system. Neighbor-joining method was used for constructing the phylogenetic tree with a bootstrap value of 1,000 replication by using MEGA 7.0 software (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Our continuous research has been carried out to isolate the marine fungi, especially those from marine sponges. The

screening of antimicrobial and anticancer activities of fungi extracts showed that it can be exploited as alternative sources for biomedical therapy (Artasasta *et al*., 2018; Handayani and Aminah, 2017; Handayani and Artasasta, 2017; Handayani *et al*., 2016; 2018). Some marine sponge-derived fungi were proved to produce some bioactive compounds which are beneficial for health. The marine brown algal genus *Padina* are widely distributed throughout the tropics and are very easy to recognize in the field. Based on a study by Wong *et al*. (2015), endophytic fungi founded in *Padina* sp. were active against some pathogen microbes. Due to their different and unique living condition, *Padina* sp. collected from Indonesia was thought of having some different kinds of endophytic fungi which may lead to the new source of new chemical structures from marine algae. Based on antimicrobial activity that showed in Table 1, six extracts of fungi were active against *E. coli* and five extracts were active against *S. aureus* and *C. albicans* in the concentration of 5%. Based on the diameter of the inhibition zone, there were two isolates showing the promising results, Nita$_3$ (NT_3) and NT_9. The inhibition zone from the extract NT_3 and NT_9 against *S. aureus* was 17.09 and 12.8 mm, respectively. However, against *E. coli*, only NT_3 had good promising antibacterial with a diameter of inhibition zone of 13.60 mm and NT_9 had no activity. Extract NT_3 and NT_9 against pathogenic fungus, *C. albicans*, also had good promising activity with a diameter zone of 20.98 and 11.1 mm, respectively.

Compared to all endophytic fungi isolate, NT_3 was the most active against all tested pathogenic microbes. Macroscopically, NT_3 has white color at the upper and green color at the bottom, almost radial shape, and furred. Based on the molecular identification using the ITS region, the obtained DNA band was around 640 bp (Fig. 1). A BLAST search in NCBI-GenBank showed that the NT_3 isolate had a maximum identity of 99% with *T. harzianum*. Using the BOLD system, NT_3 isolate showed 99.63% similarity with *T. harzianum*. There is no enough morphological information about *T. harzianum*, but at the genus level, *Trichoderma* is known to have white, yellow, until green color, macroscopically. *Trichoderma* sp. is a

Table 1. Antimicrobial activity of EtOAc extract of endophytic fungi from marine algae *Padina* sp.

Fungi code	Zone of inhibition (mm) ± standard deviation (SD)								
	E. coli			*S. aureus*			*C. albicans*		
	1%	3%	5%	1%	3%	5%	1%	3%	5%
NT_1	-	-	-	-	7.10 ± 0.00	8.00 ± 0.00	-	7.00 ± 0.00	8.00 ± 0.00
NT_2	7.45 ± 0.00	8.75 ± 0.00	9.10 ± 0.00	-	-	-	-	-	6.70 ± 0.00
NT_3	8.07 ± 1.91	18.0 ± 1.75	20.9 ± 1.55	9.90 ± 0.35	15.4 ± 4.56	17.1 ± 6.57	-	6.35 ± 0.00	13.6 ± 0.00
NT_4	10.1 ± 2.89	7.50 ± 0.21	8.10 ± 6.57	-	-	-	-	7.00 ± 0.00	6.00 ± 0.00
NT_5	-	-	-	7.20 ± 0.00	7.55 ± 0.00	10.1 ± 0.00	-	-	-
NT_6	6.95 ± 0.00	7.50 ± 0.00	7.58 ± 0.00	-	-	-	-	-	-
NT_7	-	7.90 ± 0.00	8.50 ± 0.00	-	6.05 ± 0.00	8.47 ± 0.00	-	-	6.50 ± 0.00
NT_8	-	-	7.35 ± 0.00	-	-	-	-	-	-
NT_9	-	-	-	7.20 ± 0.00	12.4 ± 0.00	12.8 ± 0.00	-	6.60 ± 0.00	11.1 ± 0.00

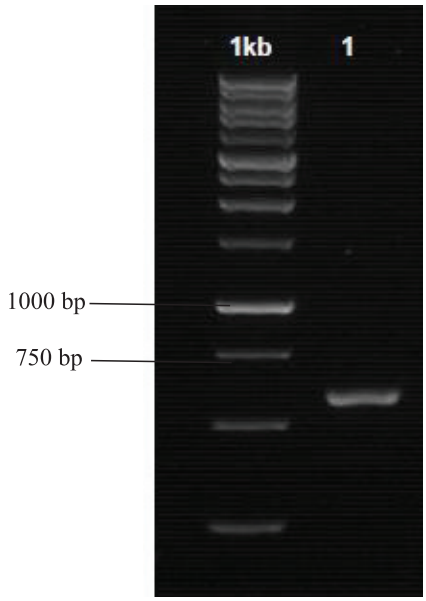

Figure 1. PCR product of ITS gene from NT3 isolate derived from Padina sp. There is a 640 bp band from the sample and the marker is 1 kb.

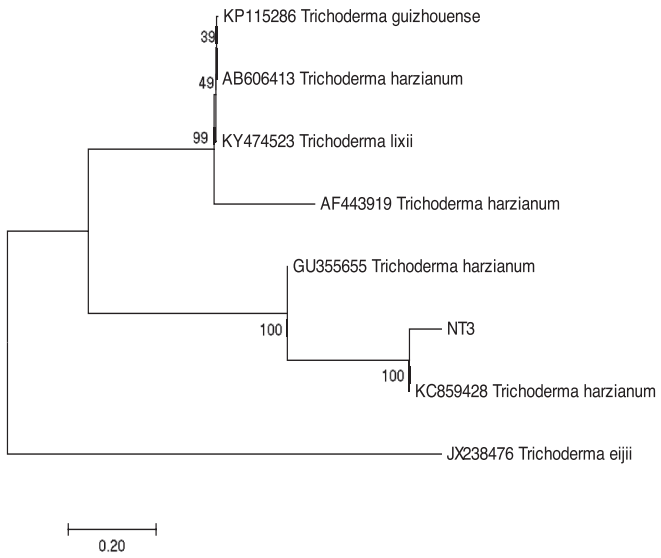

Figure 2. The phylogenetic tree inferred using the neighbor-joining method of ITS sequence of NT_3 endophytic fungal from Padina sp. and its allied taxa.

Table 2. Phytochemical screening of EtOAc extract of endophytic fungi from marine algae *Padina* sp.

Fungi code	Secondary metabolite compound		
	Alkaloid	Phenolic	Terpenoid/Steroid
NT_1	(+)	(+)	(−)
NT_2	(+)	(+)	(−)
NT_3	(−)	(−)	(−)
NT_4	(+)	(+)	(+)
NT_5	(−)	(−)	(−)
NT_6	(+)	(−)	(−)
NT_7	(+)	(+)	(+)
NT_8	(+)	(+)	(+)
NT_9	(+)	(+)	(+)

Table 3. Estimation of evolutionary divergence between sequences using the Kimura 2-parameter model.

		1	2	3	4	5	6	7	8
1	NT3								
2	KY474523 *Trichoderma lixii*	1.227							
3	KP115286 *Trichoderma guizhouense*	1.217	0.004						
4	GU355655 *Trichoderma harzianum*	0.230	0.585	0.585					
5	AB606413 *Trichoderma harzianum*	1.217	0.002	0.002	0.580				
6	AF443919 *Trichoderma harzianum*	1.111	0.233	0.236	1.223	0.236			
7	KC859428 *Trichoderma harzianum*	0.039	1.072	1.064	0.283	1.064	0.975		
8	JX238476_Trichoderma_eijii	1.904	1.473	1.488	1.657	1.473	1.655	1.805	

microorganism living in almost all kinds of soil, varied habitat, and is a saprophyte (Harman, 2000).

Neighbor-joining method was used for constructing the phylogenetic tree with a bootstrap value of 1,000 (Fig. 2). In this tree, NT_3 is clustered together with *T. harzianum* KC859428 from Kerala (India). The genetic difference between them is only 3.9% according to the Kimura 2-parameter model (Table 3). *Trichoderma harzianum* is actually synonym to *T. lixii* and *T. guizhouense* (Chaverria *et al*., 2003; 2015). Using the entire ITS region, the threshold value of 99% similarity is set for distinguishing species in a genus (Garnica *et al*., 2016). This implies that the classification of the genus *Trichoderma* needs to be revisited. Little is known about the bioactive compound of *T. harzianum* as an antibacterial agent. However, a metabolite of a genus of *Trichoderma* repeatedly shows interesting bioactivity against pathogenic bacteria. Aspereline A isolated from *Trichoderma* sp., for example, has bioactivity against *S. aureus* and *E. coli* with MIC < 100 µg/ml (Ren *et al*., 2009). In addition, Trichoderin A isolated from *Trichoderma* sp. has a potential bioactive against *Mycobacterium smegmatis, M. bovis,* and *M. tuberculosis* with MIC < 2.0 µg/ml (Pruksakorn *et al*., 2010).

Study of Ghisalberti and Rowland (1993) about anti-fungal metabolites from *T. harzianum* revealed that octaketide keto diol and octaketide-derived compounds have strong antifungal against *Rhizoctonia solani.* These compounds are polyketide group, which is responsible for their antimicrobial properties (Hermosa *et al*., 2014). Phytochemical screening of extracts from nine isolates showed alkaloid, phenolic, triterpenoid, and steroid compounds (Table 2). However, the NT_3 extract showed negative results for this test. Therefore, polyketide was estimated as a compound for providing an antimicrobial property of *T. harzianum*.

CONCLUSION

Endophytic fungal NT_3 from *Padina* sp. showed potential bioactive against several pathogenic microbes *S. aureus*, *E. coli*, and *C. albicans*. Molecular characterization indicated that NT_3 was *T. harzianum*. Further research needs to be conducted to determine the bioactive compound that has a role in inhibiting the growth of these pathogenic microbes.

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CONFLICT OF INTEREST

There is no conflict of interest.

REFERENCES

Artasasta MA, Yanwirasti DA, Handayani S. Cytotoxic activity screening of ethyl acetate fungal extracts derived from the marine sponge *Neopetrosia chaliniformis* AR-01. J Appl Pharm Sci, 2017; 7(12):174–8.

Ashok G, Senthilkumar G, Panneerselvam A. Diversity and seasonal variation of soil Fungi isolated from coastal area of Tuticorin Dt., Tamil Nadu, India. Int J Curr Microbiol App Sci, 2015; 4(10):161–78.

Atashpaz S, Khani S, Barzegari A, Barar J, Vahed S, Azarbaijani R, Omidi Y. A robust universal method for extraction of genomic DNA from bacterial species. Microbiology, 2010; 79(4):538–42.

Bacon CW, White JF. Microbial endophytic. Marcel Dekker, New York and Basel, pp 1–487, 2000.

Bauer AW, Perry DM, Kirby WMM. Single-disk antibiotic-sensitivity testing of Staphylococci: an analysis of technique and results. Arch Intern Med, 1959; 104(2):208–16.

Bugni TS, Ireland CM. Marine-derived fungi: a chemically and biologically diverse group of microorganisms. Nat Prod Rep, 2004; 21:143–63.

Chaverria P, Branco-Rocha F, Jaklitsch W, Gazis R, Degenkolb T, Samuels GJ. Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. Mycologia, 2015; 107(3):558–90.

Chaverria P, Castlebury LA, Samuels GJ, Mgeisera D. Multilocus phylogenetic structure within the *Trichoderma harzianum*/*Hypocrea lixii* complex. Mol Phylogenet Evol, 2003; 27(2):302–13; doi:10.1016/S1055-7903(02)00400-1.

Ferrer C, Colom F, Frases S, Mulet E, Abad JL, Alió JL. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. J Clin Microb, 2001; 39(8):2873–9.

Flewelling AJ, Currie J, Gray CA, Johnson JA. Endophytes from marine macroalgae: promising sources of novel natural products. Curr Sci, 2015; 109:88–111.

Garnica S, Schön ME, Abarenkov K, Riess K, Liimatainen K, Niskanen T, Dima B, Soop K, Frøslev TB, Jeppesen TS, Peintner U, Kuhnert-Finkernagel R, Brandrud TE, Saar G, Oertel B, Ammirati JF. Determining threshold values for barcoding fungi: lessons from cortinarius (Basidiomycota), a highly diverse and widespread ectomycorrhizal genus. FEMS Microbiol Ecol, 2016; 92(4):fiw045; doi:10.1093/femsec/fiw045.

Ghisalberti EL, Rowland CY. Antifungal metabolites from *Trichoderma harzianum*. J Nat Prod, 1993; 56(10):1799–804.

Handayani D, Aminah I. Antibacterial and cytotoxic activities of ethyl acetate extract of symbiotic fungi from West Sumatra marine sponge *Acanthrongylophora ingens*. J Appl Pharm Sci, 2017; 7(02):237–40.

Handayani D, Artasasta MA. Antibacterial and cytotoxic activities screening of symbiotic fungi extracts isolated from marine sponge *Neopetrosia chaliniformis* AR-01. J Appl Pharm Sci, 2017; 7(05):066–9.

Handayani D, Ornando R, Rustini R. Antimicrobial activity screening of symbiotic fungi from marine sponge *Petrosia nigrans* collected from South Coast of West Sumatera Indonesia. Int J Pharmacogn Phytochem Res, 2016; 8(4):623–6.

Handayani D, Rasyid W, Rustini, Zainudin EN, Hertiani T. Cytotoxic activity screening of fungal extracts derived from the West Sumatran marine sponge *Haliclona fascigera* to several human cell lines: Hela, WiDr, T47D, and Vero. J Appl Pharm Sci, 2018; 8(01):055–8.

Harman GE. Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzinum* T-22. Plant Dis, 2000; 84(04):377–93.

Hawksworth DI. The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycol Res, 1991; 95:641–55.

Hermosa R, Cardoza RE, Rubio MB, Gutierrez S, Monte E. Chapter 10: Secondary metabolism and antimicrobial metabolites of Trichoderma. In: Biotechnology and biology of Trichoderma. Elsevier B.V., Netherlands, pp 125–37, 2014.

Kjer J, Debbab A, Aly AH, Proksch P. Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. Nat Protoc, 2010; 5(3):479–90.

Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol, 2016; 33(7):1870–4.

Pruksakorn P, Arai M, Kotoku N, Vilcheze C, Baughn AD, Moodley P, Jacobs WR Jr, Kobayashi M. Trichoderins, novel aminolipoptides from marine sponge-derived *Trichoderma sp.* Are active against dormant mycobacteria. Bior Medi Chem Lett, 2010; 20:3658–63.

Rateb ME, Ebel R. Secondary metabolites of fungi from marine habitats. Nat Prod Rep, 2011; 28:290–344.

Ren J, Xue C, Tian L, Xu M, Chen J, Deng Z, Proksch P, Wenhan L. Asperelines A-F, peptaibols from the marine-derived fungus *Trichoderma asperellum*. J Nat Prod, 2009; 72(6):1036–44.

Singh RP, Kumari P, Reddy CRK. Mini review: Antimicrobial compounds from seaweeds-associated bacteria and fungi. Appl Microbiol Biotechnol, 2014; 99(4):1571–86; doi:10.1007/s00253-014-6334-y.

Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev, 2003; 67:491–502.

Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction: a review. Int Pharm Sci, 2011; 1(1):98–106.

Wong C, Proksch P, Tan LT, Lihan S, Mujahid A, Müller M. Isolation, identification and screening of antimicrobial properties of the marine-derived endophytic fungi from marine brown seaweed. Microbiol Indones, 2015; 9(4):141–9.

Zhang P, Mandi A, Li XM, Du FY, Wang JN, Li X, Kurtan T, Wang BG. Varioxepine A, a 3H-Oxepine-containing alkaloid with a new oxacage from the marine algal-derived endophytic fungus *Paecilomyces variotii*. Org Lett, 2014; 1(2):A–D.

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